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CANHAM — Atty. Ref.: 124-822

Appl. No. 09/743,447 — Group: 1744

Filed: January 10, 2001 — Examiner: Beisner

For: TRANSFERRING MATERIALS INTO CELLS USING POROUS SILICON

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION OF PRIOR INVENTION UNDER 37 C.F.R. §1.131

I, Leigh Trevor Canham, a British subject, hereby declare as follows:

1. That I am the inventor of the above-identified application and I reside in Malvern, Great Britain.

2. That subsequent to January 1, 1996 and prior to June 10, 1998, I conceived the subject matter of the present invention in Great Britain, a WTO member country.

3. That prior to June 10, 1998 I prepared an invention report, a true and correct copy of which is attached as Exhibit A, entitled "DNA transfer into cells using micromachining and porous silicon technology" describing my invention and reporting my invention to my employer.

4. That prior to June 10, 1998 I received, read and understood a first draft of a patent application prepared by a United Kingdom patent attorney advising my employer, a true and correct copy of which is attached as Exhibit B.

5. That prior to June 10, 1998 I received, read and understood a second draft of a patent application also prepared by a United Kingdom patent attorney advising my employer, a true and correct copy of which is attached as Exhibit C.

6. That dates on each of Exhibits A, B and C have been redacted, but all of them are prior to June 10, 1998.

That I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 6th August 2003.

Leigh Trevor Canham

Sent for Approval:

DRAFT DESCRIPTION

- on -

TRANSFERRING MATERIALS INTO CELLS AND A MICRONEEDLE ARRAY

- of -

THE SECRETARY OF STATE FOR DEFENCE

Ref: J1446

TRANSFERRING MATERIALS INTO CELLS AND A MICRONEEDLE ARRAY

This invention relates to ways of transferring materials into cells, and also to a microneedle array.

There are many times when it is necessary to transfer materials into cells, for example nucleic acids or nucleic acid constructs, such as vectors or plasmids, etc. have to be transferred into a cell for the purposes of genetic manipulation. Furthermore, chemicals may also need to be transferred into cells, e.g. nucleotides or stains, and chemicals to affect the physiology of a cell. A number of chemical and mechanical processes have been developed to convey materials into cells. These techniques include:-

1. direct microinjection - a needle is inserted into a cell and material expelled through the needle;

2. electroporation - the cell membrane is made permeable to some molecules by application of a high voltage shock;

3. biolistics - tungsten or gold particles are coated with the substance desired to be introduced and are shot into the cell;

4. calcium phosphate co-precipitation - cells absorb calcium phosphate, and if DNA/other material co-precipitates with the calcium phosphate it is also taken into the cell;

5. mediated transformation (via liposome, viral, or bacterial vectors); and

6. protoplast transformation.

An aim of one aspect of the present invention is to use a new material to assist in the transfer of substances to cells.

An aim of another aspect of the invention is to provide an improved way of providing small volumes of a substance.

Direct microinjection involves the insertion by a microneedle of DNA directly into the nucleus of individual cells. A glass micropipette linked to a micromanipulator is used to inject 10^{-8} - 10^{-7} µl typically a solution of DNA fragments into cell nuclei. "Hits" are almost certain, given considerable operator expertise but the technique is laborious and cannot be applied to a large number of cells.

According to a first aspect the invention comprises a cell-penetrating member made of porous silicon.

The cell-penetrating member is adapted to have a substance to be introduced into a cell carried by the porous silicon.

It has been discovered that porous silicon is biocompatible, and it has now been discovered that porous silicon can be corroded in, or resorbed into, a mammalian body without significant detrimental effect. Porous silicon can be used to locate and mount biological material (or any substance to be introduced into a cell).

It is known from PCT Patent Application No. PCT/US95/12381 to have an array of micromachined bulk silicon barbs or tips and to use them mechanically to pierce the plasma membrane of large numbers of cells

simultaneously. This is more efficient than piercing a single cell with a single needle, which can result in a laborious operation if hundreds of cells need to have material introduced into them. The tips of PCT/US95/1281 are, with hindsight, less effective at transferring material (e.g. DNA) into a pierced cell than they might be. It is, for example, proposed in that document to use surface tension forces between closely-spaced tips to hold biological material to be introduced into the cells in the spaces between the tips, and to trap it between the tips (probes) and the substrate.

According to a second aspect, the invention comprises a micropiercer comprising at least a region of porous silicon.

The porous silicon region is adapted to immobilise a substance (e.g. DNA). The porous silicon region is preferably at the tip of the micropiercer. The micropiercer may be a tip or barb, with no central lumen, or it may be a needle with a central channel. The micropiercer may have a pore network extending from a reservoir or channel to a substance delivery region provided on the surface of the micropiercer.

The micropiercer may have a coating of porous silicon, or it may be porous throughout its cross-section, at least at its tip (or other substance delivery region if that is not the tip). Substantially the whole exterior surface of the micropiercer that penetrates a cell in use may comprise porous silicon. The micropiercer may be a bulk silicon microtip with a porous silicon coating.

An advantage of holding the substance to be introduced to the cell at the tip of the micropiercer itself, instead of in channels/spaces between tips, is that the material is definitely introduced into the cell, and typically deeply into the cell. This may increase the success rate of the operation (in

many cases introducing DNA into cells and stable uptake of the DNA/fragment is not statistically very successful - a few percent may succeed, which is why so many cells have to be injected).

Instead of using porous silicon to immobilise the material on the tip/ensure at least some material is present on the tip, other holding means may be used. For example, polycrystalline silicon can hold some substances at grain boundaries. The holding means may comprise a porous material.

It is known to immobilise DNA fragments in ~~micro~~macro porous silicon in the field of a flow-through genosensor (Advances in Genosensor Research. K.L. Beattie et al. Clin. Chem. 41, 700 (1995)).

An advantage of porous silicon is that its bioactivity can be tuned by controlling its pore size and porosity. It is therefore possible to create a micropiercer with a porous tip with pores tailored to hold/immobilise a particular desired molecule or substance. Of course, the substance will not be so immobilised that at least some of the material cannot leave the tip when the tip is in the cell.

Porous silicon has another great advantage as the choice of material for a micropiercer in that micromachining techniques for fabricating small scale devices from silicon exist, e.g. in the electronics industry.

It is known how to make a silicon structure porous (see for example US 5 348 618, the contents of which are hereby incorporated by reference).

An array of micropiercers may be provided.

It is also known to have an array of microtips for a completely different purpose - for field emission cathodes used in vacuum microelectronic applications. Here, a 5mm square silicon chip will typically contain about 500 microtips of pyramidal shape with tip widths of 50mm - 1μm and heights of 10 - 100μm, depending upon the manufacturing parameters chosen. With hindsight, these would be suitable for porosification and then use as micropiercers for transferring a substance into cells. It is also even known to have porous silicon pyramidal cathodes - e.g. Field emission from pyramidal cathodes covered in porous silicon. P.R. Wilshaw et al. J. Vac. Sci. Techn. B12,1 (1994); Fabrication of Si field emitters by forming porous silicon. D. Kim et al. J. Vac. Sci. Tech. B14, 1906 (1996); and Porous silicon field emission cathode development. J.R. Jessing et al. J. Vac. Sci. Techn. B14, 1899 (1996). However, these are all in a totally different field, and none show a micropiercer having held on it DNA, RNA, or any other substance to be introduced into a cell.

According to a third aspect, the invention comprises a method of producing a micropiercer device comprising manufacturing one or more micropiercer projections, and providing substance holding means at or near the tip of the projections.

Preferably the method comprises making at least a part of the projections porous. Preferably the method comprises making the tip of the projection porous, or providing a porous coating on the tip. Preferably the tip is made porous using an HF anodising technique.

According to another aspect, the invention comprises a method of transferring a material into a cell comprising associating the material with a tip portion of a micropiercer and piercing the cell with the micropiercer.

Preferably the method comprises using porous silicon to locate the material at or near the tip portion.

According to a further aspect, the invention comprises a method of genetic manipulation of a cell comprising associating genetic material with a tip portion of a micropiercer, piercing the cell with the micropiercer to allow the genetic material to enter the cell. The genetic material may then be stably incorporated in the cell.

According to another aspect, the invention comprises a microneedle array comprising a plurality of needles extending away from a support, the needles each having fluid transport means adapted to transport fluid from their bases to their tips, and fluid supply means communicating with the fluid transport means and adapted to supply fluid to be injected to the base of the needles.

Preferably the array of microneedles are made of silicon. It may be micromachined, for example from a silicon wafer.

The fluid transport means may comprise a reservoir, which may extend under the needles. The device may comprise a body having a lower portion, an upper portion, and a channel or reservoir extending between the upper and lower portions, with the needles being provided in the upper portion and the fluid transport means extending to the reservoir or channel.

The fluid transport means may comprise a single lumen, or macropore in each needle which may extend generally centrally of the

needle. Alternatively, or additionally, the fluid transport means may comprise a pore or capillary network, such as a plurality of mesopores.

The array of needles may be provided on an integrated silicon chip, which may also have ~~XXXXXX~~ a sensor provided on it, preferably the sensor enabling one to monitor in situ the transfection process. For example a photo emitter/detector may be used in association with light emitting markers associated with the DNA.

What else will the chip have on it/might it have on it? - ask client - e.g. power source; sensor?; light emitter?; processing circuitry/control circuitry? What is on the chip?

It may also be desirable to have a power supply, and/or processing circuitry, and/or control circuitry provided on the chip. Arrays of light emitting devices and photodetectors may enable the transfection process to be monitored under high spatial resolution.

According to another aspect, the invention comprises a method of manufacturing a microneedle, or a microneedle array, the method comprising taking a bulk silicon wafer and creating a needle or an array of needles; and creating fluid transfer means extending from the base of the or each needle to its tip.

Preferably, the method comprises providing a network of pores from the base of the or each needle to its tip. The pores may be macropores or mesopores, or for some applications they may even be micropores (but macropores are preferred).

The or each needle may be created using photolithographic techniques such as anisotropic etching and photo-resist lithographic techniques.

The silicon substrate may be an n-type substrate with a resistivity in the range of 0.1-10Ωcm.

When the tips/needles have been created it may be desirable to in-fill ~~[WHAT?]~~ and planarise, for example using a non-conducting mask material, ~~[WHY? WHY IS IT DESIRABLE?]~~

The in-filled and planarised array may then be treated so as to expose just the tips, for example by using an oxygen plasma treatment and an HF dip to expose the tips alone. The tips can then be anodised to create the macropores from the tip to the wafer back surface. The wafer, provided with an array of tips, may then be bonded to another backing member, which may be shaped so as to define a channel or reservoir between the tip-carrying wafer and the backing member.

According to a further aspect the invention comprises a vehicle for transferring material into a cell, the vehicle comprising at least in part resorbable material.

Preferably the vehicle comprises resorbable silicon, such as porous silicon, or polycrystalline silicon. The whole of the vehicle may be made of the resorbable material, or only part of it. The vehicle may comprise bioactive silicon. (By "resorbable" it is meant that the material is corroded/absorbed/eroded/or otherwise disappears when in situ in physiological fluids. By "bioactive" it is meant that the material can induce the deposition of Calcium Phosphate precipitates on its surface under physiological conditions in body fluid.

If the vehicle is retained in the cell it will be adsorbed/corroded/eroded or resorbed, or partially resorbed, and be less of an irritation/foreign body to the cell in due course.

The resorbable silicon/other material may be used in a biolistics technique.

The vehicle for transferring material into the cell may comprise a biolistic bullet comprising porous silicon.

The bullet may have a substance to be introduced into a cell adhered to it. The bullet may be impregnated with material (e.g. DNA material). It may be substantially saturated with material. The bullet may comprise a submicron silicon particle. The silicon particle may be rendered porous by stain etching techniques. The particle is preferably mesoporous.

A resorbable biolistic bullet would not leave behind in the cell a particle, as do gold or tungsten biolistic bullets. The bullet need not be porous all of the way through - it may have a porous coating. The resorbable bullet need not necessarily be made of porous silicon, or of silicon at all, but porous silicon has been identified as an especially suitable material.

According to another aspect, the invention provides a method of transferring material into a cell comprising the steps of shooting a vehicle carrying said material into the cell.

Preferably the vehicle is the vehicle as hereinabove defined. Preferably the bullet is shot into the cell be means of a pressurised gas, for example helium.

The process of biological biolistics is often used where more standard techniques do not work. Resorbable impregnated materials, such as porous silicon offer biocompatible advantages over corrosion-resistant bulk metal materials.

According to a further aspect the present invention provides a method of making a vehicle for transferring material into a cell comprising

the steps of rendering the vehicle at least partially porous and introducing to the vehicle the material to be transferred to the cell.

Preferably the vehicle comprises a silicon bullet, most preferably a submicron silicon particle, which may be rendered porous, preferably mesoporous by stain etching techniques. The bullet may have the material to be introduced to the cell adhered to it or alternatively it may be impregnated with the material.

The vehicle for transferring material into a cell may comprise bioactive silicon. It may comprise a bioactive silicon particle having the material to be transferred in a form adapted to co-precipitate with a substance which is taken up by cells. The co-precipitate may be a calcium phosphate precipitate.

According to another aspect the invention comprises a method of introducing material into a cell comprising associating the material with a silicon particle, precipitating calcium phosphate onto the particle to form a calcium phosphate/silicon particle combined particle, and arranging for the cell to uptake the calcium phosphate/silicon particle combination.

In the technique of electroporation the cell membrane can be made permeable by exposing cells to a brief electric shock of very high voltage. Low porosity bioactive silicon is electrically conducting and is suitably developed as an intimate coupling matrix for adherent mammalian cells growing on microelectrode arrays.

By having bioactive silicon, e.g. porous silicon or polycrystalline silicon, as one or both electrodes in electroporation apparatus it is envisaged that better DNA transfer takes place.

According to a further aspect the invention comprises a method of electroporation comprising providing an electrically conducting bioactive silicon electrode.

Preferably the method comprises growing cells on the electrode. The method may comprise providing an array of bioactive silicon electrodes, possibly with cells grown on them. The electrode, or electrodes, may be coated with porous silicon or may be of porous silicon throughout their cross-section, at least at a region of their height.

According to a further aspect the invention comprises electroporosis apparatus comprising a bioactive electrode. Preferably the electrode is bioactive silicon, most preferably porous silicon. An array of electrodes, or microelectrodes, may be provided.

The electrodes may have a length of XXXX (range - what length?) and a width of XXXX (range - what width?).

The invention may also reside in the use of bioactive silicon, preferably porous silicon, in the preparation of apparatus for the introduction of materials into cells.

Several embodiments of the present invention will now be described by means of example only with reference to the Figures, in which:-

Figure 1 shows a partially porosified silicon microtip;

Figure 2 shows a silicon microneedle array;

Figure 3 shows the silicon microneedle array of Figure 2 having a macroporous network running from the tip to an underlying reservoir;

Figure 4 shows a porous silicon bullet impregnated with DNA;

Figure 5 shows a porous silicon core impregnated with DNA and surrounded by calcium phosphate; and

Figure 6 illustrates the electroporation technique of the present invention.

Figure 1 shows a micropiercer 10 in the form of a microtip 12 having a base width A of 50μm and a height B of 100μm and a tip width C of 0.5μm. The surface of the microtip 12 is coated with porous silicon 14 having a depth D of 0.1μm.

In use, the porous silicon coating 14 immobilises the substance to be delivered to the cell (e.g. DNA/RNA) on the tip itself, which increases the chances of the immobilised substance on the tip being introduced into the cell.

The pore size and porosity of the porous silicon coating can be controlled to tune the bioactivity of the microtip 12. By controlling the pore size and porosity of the porous silicon, we can make particular molecules come off it more, or less, readily. We may leave the microtips inside a cell for a predetermined time to allow molecules to disassociate themselves from the porous silicon.

Figure 2 shows an array 20 of silicon microneedles 22 extending away from a silicon support, or back, member 24. The microneedles 22 have porous silicon microtips 26 and a central lumen 28 communicating between the microtips 26 and a reservoir 30 defined between an upper member 32, provided with the microneedles 22 and the back, support, member 24. The back member 24 is of bulk silicon.

Figure 3 shows an array 33 of silicon microneedles 34 that is similar to that of Figure 2. The principal difference between the arrays shown in Figures 2 and 3 is that the microneedles 34 shown in Figure 3 are not provided with a central lumen 28. Instead the array 33 of silicon microneedles 34 in Figure 3 is provided with a mesoporous network 36 which extends from the microtips of the microneedles to the reservoir 30', allowing fluid communication between the reservoir 30' and the microtips.

In use, the substance to be delivered to cells is provided to the porous silicon microtips 22,34 from the reservoir 30,30' through the central lumens 28 or the mesoporous network 36. The substance is then held by the porous silicon microtips ready for introduction into a cell.

The material to be introduced into the cells may be pumped into the reservoir 30, 30', and out through the lumens 28 or porous network by a pump, not shown (but arrow 39 indicates the pump delivering liquid to the reservoir).

All or part of the silicon surfaces within the final structure may be treated in such a way as to modify their interaction with biological systems. This might be achieved by forming a layer of porous silicon on the surface. Such a layer could be formed by either an electrochemical anodisation

process or possibly by immersing the structure into a stain etching solution such as a mixture of hydrofluoric acid and nitric acid.

Figure 4 shows a biolistic bullet 40 comprising a submicron silicon particle rendered mesoporous by stain etching.

In use, the bullet 40 is impregnated with the substance to be introduced into a cell and is shot into the cell using pressurised helium. As the porous silicon is a resorbable material, it will be preferably fully resorbed, and at least partially resorbed, by the cell that it entered, and thus comprises less of a foreign body than known biolistic bullets such as gold or tungsten which leave particles of metal in the cell.

Figure 5 shows a porous silicon core 50 impregnated with a substance to be introduced into a cell (e.g. DNA/RNA) and calcium phosphate precipitate 52 formed around the core 50. The calcium phosphate 52 is co-pecipitated with DNA/RNA, so that a genetic material/calcium phosphate layer surrounds the bioactive silicon core 50. The bioactive silicon core locally induces calcium phosphate supersaturisation. It may be possible to place a bioactive silicon core next to a cell/against the wall of a cell, and co-precipitate DNA/$Ca(PO_4)_2$ against the core and against the wall of the cell. If the core is phagocytosed it can be resorbed.

The core 50 need not have DNA/RNA/any active substance on it - it may simply serve as a good nucleation site for co-precipitation of DNA/$Ca(PO_4)_2$.

Insert re use of glass beads in calcium phosphate method. bioinert

It is known to use glass beads as a nucleation site for calcium phosphate co-precipitation DNA transfection - see for example the paper by Watson and Latchman in "Methods (San Diego) 1996 [illegible]

L-C to provide reference

It will be appreciated that micropores are pores with a diameter of 2 nm or less; mesopores have a diameter of 2nm - 50 nm; and macropores have a diameter of 50 nm or more.

It has also been realised that it is possible to improve the efficiency of the introduction of materials to cells in an electroporation technique, as shown in Figure 6, using porous silicon, preferably mesoporous silicon (but macroporous and microporous silicon are also useful).

The use of a porous silicon (or porous other bioactive material, or bioactive polycrystalline silicon) electrode 60,61 achieves better performance in electroporation. Because the electrode is bioactive, instead of being bioinert, cells (typically animal cells) have an affinity to it and are localised on its surface.

Low porosity (50% or less, or 30% or less, or 10% or less) bioactive silicon is electrically conducting and is a suitable intimate complex matrix for adherent mammalian cells 62, which may grow on a microelectrode array 60,61. Thus, it is possible to grow mammalian cells on bioactive porous silicon electrodes and then introduce DNA (or other substances) into the cells by using electroporation, with the substrate upon which the cells are grown being an electrode, or even both electrodes 60,61, of the electroporation apparatus. This has advantages in handling the cells, and achieves a better efficiency rate of DNA introduction than solely having the cells suspended in a liquid medium 63.

The fact that porous silicon is resorbable/erodable in vivo in mammals has been proved by the inventors, and this underpins some aspects of the invention. The fact that silicon can be made bioactive underpins other aspects of the invention.

CLAIMS

1. A method of manufacturing a microneedle, or a microneedle array, the method comprising taking bulk silicon and creating a needle or an array of needles; and creating fluid transfer means extending from the base of the or each needle to its tip.

2. The method of claim 1 further comprising providing a network of pores from the base of the or each needle to its tip.

3. The method of claim 2 wherein the pores are macropores.

4. The method of claim 2 wherein the pores are mesopores.

5. The method of claim 2 wherein the pores are micropores.

6. The method of any preceding claim wherein the or each needle is created using photolithographic techniques.

7. The method of claim 6 wherein the photolithographic technique is anisotropic etching.

8. The method of claim 6 or claim 7 wherein the photolithographic technique is a photoresistant lithographic technique.

9. The method of any preceding claim wherein the bulk silicon wafer is an n-type substrate with a resistivity in the range of 0.1-10Ωcm.

10. The method of any preceding claim wherein the needle or needle array is planarised.

11. The method of claim 10 wherein a non-conducting mask is used to planarise the needle or array of needles.

12. The method of claims 10 and 11 wherein the planarised needle or array is treated so as to expose just the tips.

13. The method of claim 12 wherein the treatment uses an oxygen plasma treatment and an HF dip.

14. The method of any one of claims 10 to 13 wherein the tips are anodised to create pores from the tip to a wafer back surface.

15. The method of any preceding claim wherein a wafer provided with an array of tips is bonded to a backing member.

16. The method of claim 15 wherein the backing member and/or tip-carrying wafer is shaped so as to define a channel or reservoir between the tip-carrying wafer and the backing member.

17. A method of producing a micropiercer device comprising manufacturing one or more micropiercer projections, and providing substance holding means at or near the tip of the projections.

18. The method of claim 17 further comprising making at least a part of the projections porous.

19. The method of claims 17 or 18 further comprising making the tip of the projections porous.

20. The method of any one of claims 17 to 19 further comprising providing a porous coating on the tip.

21. The method of any one of claims 18 to 20 wherein the tip is made porous using an HF anodising technique.

22. The method of claim 18 comprising making substantially the entire extent of the tips porous.

23. A method of transferring a material into a cell comprising associating the material with a tip portion of a micropiercer and piercing the cell with the micropiercer.

24. The method of claim 23 further comprising using porous silicon to locate the material at or near the tip portion.

25. A method of genetic manipulation of a cell comprising associating genetic material with a tip portion of a micropiercer and piercing the cell with the micropiercer to allow the genetic material to enter the cell.

26. A microneedle array comprising a plurality of needles extending away from a support, the needles each having fluid transport means adapted to transport fluid from their bases to their tips, and fluid supply means communicating with the fluid transport means and adapted to supply fluid to be injected to the base of the needles.

27. The array of claim 26 wherein the microneedles are made of silicon.

28. The array of claims 26 or 27 wherein the array is micromachined.

29. The array of claim 28 wherein the array is micromachined from a silicon wafer.

30. The array of any one of claims 26-29 wherein the fluid transport means comprises a reservoir.

31. The array of claim 30 wherein the reservoir extends under the needles.

32. The array of any one of claims 26 to 31 wherein the support has a lower portion, an upper portion, and a channel or reservoir extending between the upper and lower portions, with the needles being provided in the upper portion and the fluid transport means extending to the reservoir or channel.

33. The array of any one of claims 26 to 32 wherein the fluid transport means comprises a porous or capillary network provided in each needle.

34. The array of any one of claims 26 to 32 wherein each needle has a lumen extending through its longitudinal extent.

35. The method of claim 34 wherein the network comprises a plurality of mesopores.

36. The array of any one of claims 26 to 35 which is provided on an integrated silicon chip.

37. The method of claim 36 wherein the silicon chip has XXX.

38. A cell-penetrating member, or micropiercer, made of porous silicon.

39. The cell-penetrating member of claim 38 wherein the member is adapted to have a substance to be introduced into a cell carried by the porous silicon.

40. The cell-penetrating member of claim 42 wherein the porous silicon region immobilises a substance in comparison with its mobility when provided a bioinert substance such as titanium.

41. The cell-penetrating member of claims 39 or 40 wherein the porous silicon region is at the tip of the member.

42. The cell-penetrating member of claims 39 to 41 which has a tip or barb with no single central lumen.

43. The cell-penetrating member of claims 39 to 41 which has a needle with a central channel.

44. The cell-penetrating member of any one of claims 39 to 43 further having a pore network extending from a reservoir or channel to a substance delivery region provided on the surface of the micropiercer.

45. The cell-penetrating member of any one of claims 39 to 44 having a coating of porous silicon.

46. The cell-penetrating member of any one of claims 39 to 44 which is porous throughout its cross-section, at least at its tip.

47. The cell-penetrating member of any one of claims 39 to 46 which comprises a bulk silicon microtip with a porous silicon coating.

48. A vehicle for transferring material into a cell, the vehicle comprising at least, in part, resorbable material.

49. The vehicle of claim 48 wherein the vehicle comprises resorbable silicon.

50. The vehicle of claims 48 or 49 wherein the resorbable material is porous silicon.

51. The vehicle of claims 48 or 49 wherein the resorbable material is polycrystalline silicon.

52. The vehicle of claims 48 to 51 wherein the whole of the vehicle is made of the resorbable material, or only part of it.

53. The vehicle of claims 48 to 52 which comprises a biolistic bullet.

54. The vehicle of claim 53 wherein the bullet has a material to be introduced into a cell adhered to it.

55. The vehicle of claim 53 wherein the bullet is impregnated with material to be introduced into a cell.

56. The vehicle of claim 54 or 55 which is substantially saturated with material.

57. The vehicle of any one of claims 53 to 56 wherein the bullet comprises a submicron silicon particle.

58. The vehicle of claims 53 to 57 wherein the bullet has a porous coating.

59. The vehicle of any one of claims 53 to 58 which comprises bioactive silicon.

60. The vehicle of any one of claims 48 to 52 which has associated with it material to be transferred into a cell in a form adapted to co-precipitate with a substance which is taken up by cells.

61. The vehicle of claim 60 wherein the co-precipitate is a calcium phosphate co-precipitate.

62. A method of making a vehicle for transferring material into a cell comprising the steps of rendering the vehicle at least partially porous and introducing to it, or into it, the material to be transferred to the cell.

63. The method of claim 62 wherein the vehicle is a silicon bullet.

64. The method of claim 62 wherein the material to be transferred to the cell is adhered to the vehicle.

65. The method of claim 64 wherein the vehicle is a submicron particle and the material to be transferred to the cell is co-precipitated (with a precipitate substance), using the vehicle as a nucleation site.

66. The method of claim 62 wherein the vehicle is impregnated with the material to be transferred to the cell.

67. A method of electroporation comprising providing an electrically conducting bioactive silicon electrode.

68. A method according to claim 67 comprising growing cells on the electrode.

69. A method according to claim 67 or 68 comprising providing an array of bioactive silicon electrodes.

70. A method according to claim 69 comprising growing cells on the array.

71. Electroporation apparatus comprising a bioactive electrode.

72. Electroporation apparatus according to claim 71 in which the electrode comprises porous silicon.

73. Electroporation apparatus according to either of claims 71 or 72 having an array of electrodes, or microelectrodes.

74. A method of manufacturing the microneedle or microneedle array substantially as described herein with reference to Figure 2 or Figure 3 of the accompanying drawings.

75. A method of producing the micropiercing device substantially as described herein and with reference to Figure 1 of the accompanying drawings.

76. A cell penetration member substantially as described herein and with reference to Figure 1 or Figure 2 or Figure 3 of the accompanying drawings.

77. A vehicle for delivering material into a cell substantially as described herein with reference to Figure 4 or Figure 5 of the accompanying drawings.

78. A method of transferring material into a cell substantially as described herein with reference to Figure 4 or Figure 5 of the accompanying drawings.

79. A method of transferring material into a cell substantially as described herein with reference to Figure 6 of the accompanying drawings.

ABSTRACT

TRANSFERRING MATERIALS INTO CELLS AND A MICRONEEDLE ARRAY

The present invention relates to the use of porous silicon in the delivery of substances into cells. The porous silicon can be formed into micropiercers, microneedles and biolistic bullets for penetration of the cell. The control of the pore size and porosity of the porous silicon allows tuning of the bioactivity of the porous silicon. The porous silicon is also resorbable and is therefore resorbed from the cells without leaving any particles or being seen as a foreign body. The present invention also relates to the methods of manufacturing the porous silicon micropiercers, microneedles, microelectrodes and biolistic bullets and their advantages over known methods of delivering materials into cells.

To be accompanied, when published, by Figure 1 of the accompanying drawings.

Figure 1.

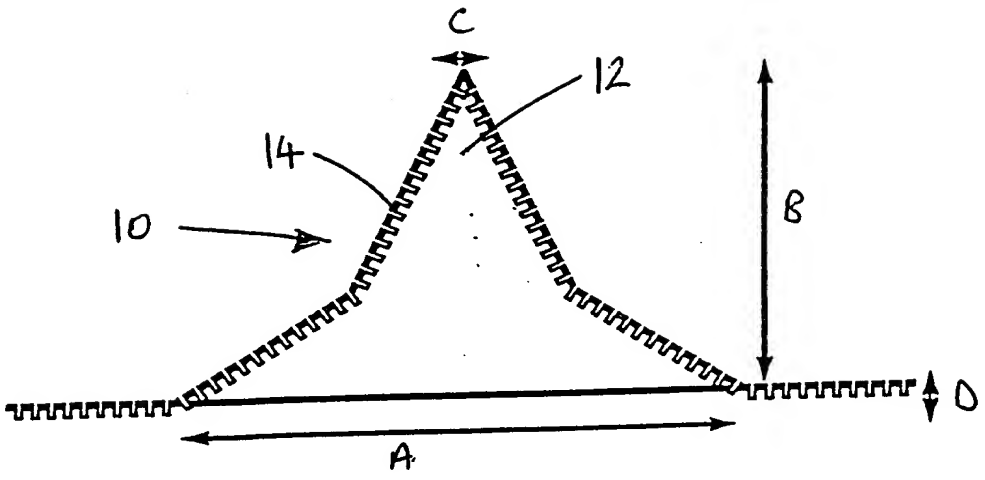


Figure 2.

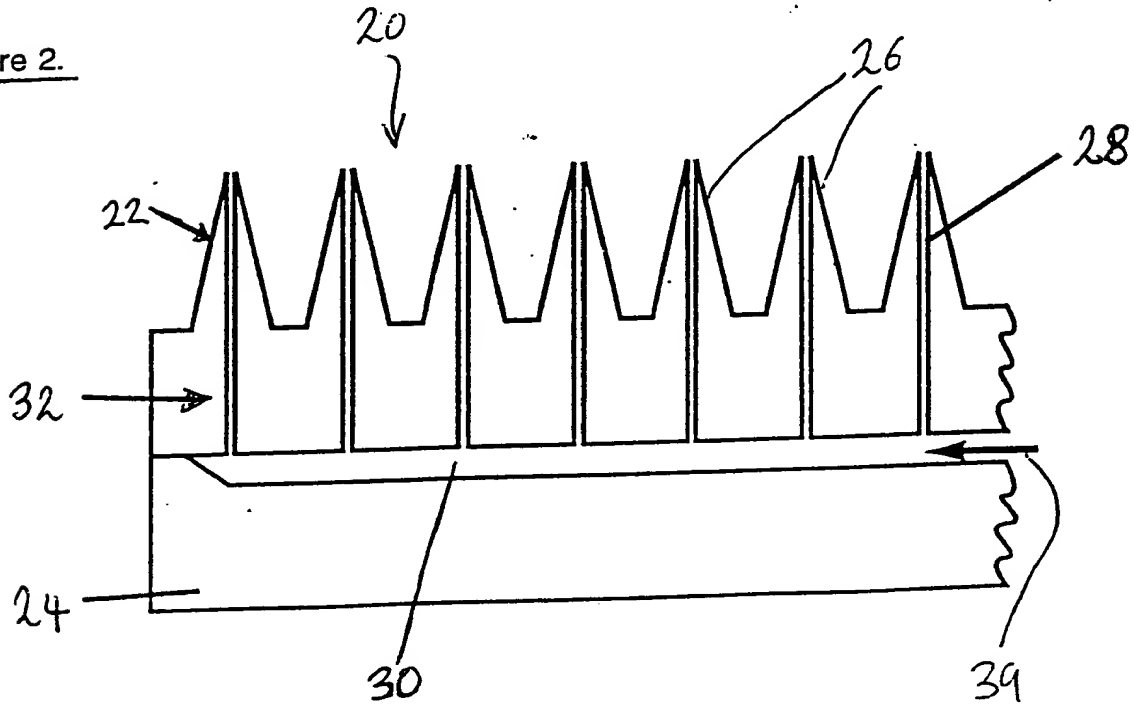


Figure 3.

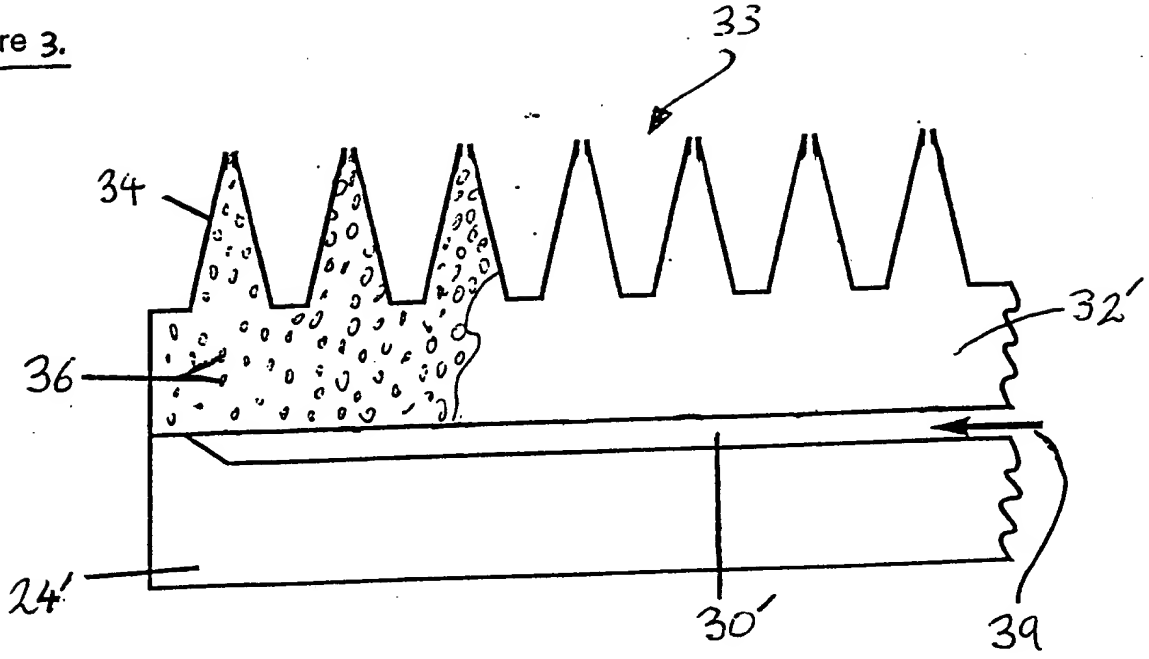
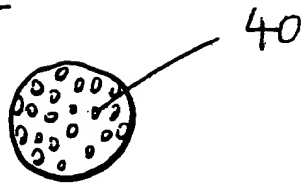


FIGURE 4.



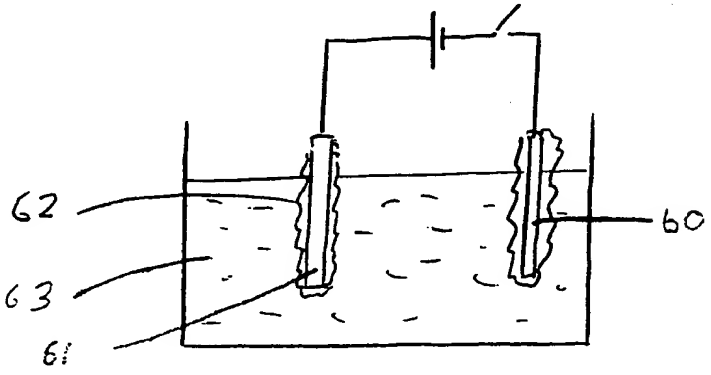


FIGURE 5.

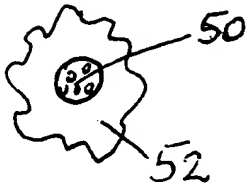


FIG. 6

Sent for Approval

DRAFT SPECIFICATION

(2nd Draft)

- on -

TRANSFERRING MATERIALS INTO CELLS AND A MICRONEEDLE ARRAY

- of -

THE SECRETARY OF STATE FOR DEFENCE

Based on:
Dated:

Ref: J1446

TRANSFERRING MATERIALS INTO CELLS AND A MICRONEEDLE ARRAY

This invention relates to ways of transferring materials into cells, and also to a microneedle array.

There are many times when it is necessary to transfer materials into cells, for example nucleic acids or nucleic acid constructs, such as vectors or plasmids, etc. have to be transferred into a cell for the purposes of genetic manipulation. Furthermore, chemicals may also need to be transferred into cells, e.g. nucleotides or stains, and chemicals to affect the physiology of a cell. A number of chemical and mechanical processes have been developed to convey materials into cells. These techniques include:-

1. direct microinjection - a needle is inserted into a cell and material expelled through the needle;

2. electroporation - the cell membrane is made permeable to some molecules by application of a high voltage shock;

3. biolistics - tungsten or gold particles are coated with the substance desired to be introduced and are shot into the cell;

4. calcium phosphate co-precipitation - cells absorb calcium phosphate, and if DNA/other material co-precipitates with the calcium phosphate it is also taken into the cell;

5. mediated transformation (via liposome, viral, or bacterial vectors); and

6. protoplast transformation.

An aim of one aspect of the present invention is to use a new material to assist in the transfer of substances to cells.

An aim of another aspect of the invention is to provide an improved way of providing small volumes of a substance.

Direct microinjection involves the insertion by a microneedle of DNA directly into the nucleus of individual cells. A glass micropipette linked to a micromanipulator is used to inject 10^{-8} - 10^{-7} µl typically a solution of DNA fragments into cell nuclei. "Hits" are almost certain, given considerable operator expertise but the technique is laborious and cannot be applied to a large number of cells.

According to a first aspect the invention comprises a cell-penetrating member made of porous silicon.

The cell-penetrating member is adapted to have a substance to be introduced into a cell carried by the porous silicon.

It has been discovered that porous silicon is biocompatible, and it has now been discovered that porous silicon can be corroded in, or resorbed into, a mammalian body without significant detrimental effect. Porous silicon can be used to locate and mount biological material (or any substance to be introduced into a cell).

It is known from PCT Patent Application No. PCT/US95/12381 to have an array of micromachined bulk silicon barbs or tips and to use them mechanically to pierce the plasma membrane of large numbers of cells

simultaneously. This is more efficient than piercing a single cell with a single needle, which can result in a laborious operation if hundreds of cells need to have material introduced into them. The tips of PCT/US95/1281 are, with hindsight, less effective at transferring material (e.g. DNA) into a pierced cell than they might be. It is, for example, proposed in that document to use surface tension forces between closely-spaced tips to hold biological material to be introduced into the cells in the spaces between the tips, and to trap it between the tips (probes) and the substrate.

According to a second aspect, the invention comprises a micropiercer comprising at least a region of porous silicon.

The porous silicon region is adapted to immobilise a substance (e.g. DNA). The porous silicon region is preferably at the tip of the micropiercer. The micropiercer may be a tip or barb, with no central lumen, or it may be a needle with a central channel. The micropiercer may have a pore network extending from a reservoir or channel to a substance delivery region provided on the surface of the micropiercer.

The micropiercer may have a coating of porous silicon, or it may be porous throughout its cross-section, at least at its tip (or other substance delivery region if that is not the tip). Substantially the whole exterior surface of the micropiercer that penetrates a cell in use may comprise porous silicon. The micropiercer may be a bulk silicon microtip with a porous silicon coating.

An advantage of holding the substance to be introduced to the cell at the tip of the micropiercer itself, instead of in channels/spaces between tips, is that the material is definitely introduced into the cell, and

typically deeply into the cell. This may increase the success rate of the operation (in many cases introducing DNA into cells and stable uptake of the DNA/fragment is not statistically very successful - a few percent may succeed, which is why so many cells have to be injected).

Instead of using porous silicon to immobilise the material on the tip/ensure at least some material is present on the tip, other holding means may be used. For example, polycrystalline silicon can hold some substances at grain boundaries. The holding means may comprise a porous material.

It is known to immobilise DNA fragments in macroporous silicon in the field of a flow-through genosensor (Advances in Genosensor Research. K.L. Beattie et al. Clin. Chem. 41, 700 (1995)).

An advantage of porous silicon is that its bioactivity can be tuned by controlling its pore size and porosity. It is therefore possible to create a micropiercer with a porous tip with pores tailored to hold/immobilise a particular desired molecule or substance. Of course, the substance will not be so immobilised that at least some of the material cannot leave the tip when the tip is in the cell.

Porous silicon has another great advantage as the choice of material for a micropiercer in that micromachining techniques for fabricating small scale devices from silicon exist, e.g. in the electronics industry.

It is known how to make a silicon structure porous (see for example US 5 348 618, the contents of which are hereby incorporated by reference).

An array of micropiercers may be provided.

It is also known to have an array of microtips for a completely different purpose - for field emission cathodes used in vacuum microelectronic applications. Here, a 5mm square silicon chip will typically contain about 500 microtips of pyramidal shape with tip widths of 50mm - 1μm and heights of 10 - 100μm, depending upon the manufacturing parameters chosen. With hindsight, these would be suitable for porosification and then use as micropiercers for transferring a substance into cells. It is also even known to have porous silicon pyramidal cathodes - e.g. Field emission from pyramidal cathodes covered in porous silicon. P.R. Wilshaw et al. J. Vac. Sci. Techn. B12,1 (1994); Fabrication of Si field emitters by forming porous silicon. D. Kim et al. J. Vac. Sci. Tech. B14, 1906 (1996); and Porous silicon field emission cathode development. J.R. Jessing et al. J. Vac. Sci. Techn. B14, 1899 (1996). However, these are all in a totally different field, and none show a micropiercer having held on it DNA, RNA, or any other substance to be introduced into a cell.

According to a third aspect, the invention comprises a method of producing a micropiercer device comprising manufacturing one or more micropiercer projections, and providing substance holding means at or near the tip of the projections.

Preferably the method comprises making at least a part of the projections porous. Preferably the method comprises making the tip of the projection porous, or providing a porous coating on the tip. Preferably the tip is made porous using an HF anodising technique.

According to another aspect, the invention comprises a method of transferring a material into a cell comprising associating the material with a tip portion of a micropiercer and piercing the cell with the micropiercer.

Preferably the method comprises using porous silicon to locate the material at or near the tip portion.

According to a further aspect, the invention comprises a method of genetic manipulation of a cell comprising associating genetic material with a tip portion of a micropiercer, piercing the cell with the micropiercer to allow the genetic material to enter the cell. The genetic material may then be stably incorporated in the cell.

According to another aspect, the invention comprises a microneedle array comprising a plurality of needles extending away from a support, the needles each having fluid transport means adapted to transport fluid from their bases to their tips, and fluid supply means communicating with the fluid transport means and adapted to supply fluid to be injected to the base of the needles.

Preferably the array of microneedles are made of silicon. It may be micromachined, for example from a silicon wafer.

The fluid transport means may comprise a reservoir, which may extend under the needles. The device may comprise a body having a lower portion, an upper portion, and a channel or reservoir extending between the upper and lower portions, with the needles being provided in the upper portion and the fluid transport means extending to the reservoir or channel.

The fluid transport means may comprise a single lumen, or macropore in each needle which may extend generally centrally of the needle. Alternatively, or additionally, the fluid transport means may comprise a pore or capillary network, such as a plurality of mesopores.

The array of needles may be provided on an integrated silicon chip, which may also have a sensor provided on it, the sensor preferably enabling one to monitor in situ the transfection process. For example a photo emitter/detector may be used in association with light emitting markers (e.g. fluorescent) associated with the DNA. It may also be desirable to have a power supply and/or processing circuitry, and/or control circuitry provided on the chip. Arrays of light emitting devises and photodetectors may enable the transfection process to be monitored under high spacial resolution.

According to another aspect, the invention comprises a method of manufacturing a microneedle, or a microneedle array, the method comprising taking a bulk silicon wafer and creating a needle or an array of needles; and creating fluid transfer means extending from the base of the or each needle to its tip.

Preferably, the method comprises providing a network of pores from the base of the or each needle to its tip. The pores may be macropores or mesopores, or for some applications they may even be micropores (but macropores are preferred).

The or each needle may be created using photolithographic techniques such as anisotropic etching and photo-resist lithographic techniques.

The silicon substrate may be an n-type substrate with a resistivity in the range of 0.1-10Ωcm.

The in-filled and planarised array may then be treated so as to expose just the tips, for example by using an oxygen plasma treatment and an HF dip to expose the tips alone. The tips can then be anodised to create the macropores from the tip to the wafer back surface. The wafer, provided with an array of tips, may then be bonded to another backing member, which may be shaped so as to define a channel or reservoir between the tip-carrying wafer and the backing member.

According to a further aspect the invention comprises a vehicle for transferring material into a cell, the vehicle comprising at least in part resorbable material.

Preferably the vehicle comprises resorbable silicon, such as porous silicon, or polycrystalline silicon. The whole of the vehicle may be made of the resorbable material, or only part of it. The vehicle may comprise bioactive silicon. (By "resorbable" it is meant that the material is corroded/absorbed/eroded/ or otherwise disappears when in situ in physiological fluids. By "bioactive" it is meant that the material can induce the deposition of calcium phosphate precipitates on its surface under physiological conditions (when in body fluids).

If the vehicle is retained in the cell it will be adsorbed/corroded/eroded or resorbed, or partially resorbed, and be less of an irritation/foreign body to the cell in due course.

The resorbable silicon/other material may be used in a biolistics technique.

The vehicle for transferring material into the cell may comprise a biolistic bullet comprising porous silicon.

The bullet may have a substance to be introduced into a cell adhered to it. The bullet may be impregnated with material (e.g. DNA material). It may be substantially saturated with material. The bullet may comprise a submicron silicon particle. The silicon particle may be rendered porous by stain etching techniques. The particle is preferably mesoporous.

A resorbable biolistic bullet would not leave behind in the cell a particle, as do gold or tungsten biolistic bullets. The bullet need not be porous all of the way through - it may have a porous coating. The resorbable bullet need not necessarily be made of porous silicon, or of silicon at all, but porous silicon has been identified as an especially suitable material.

According to another aspect, the invention provides a method of transferring material into a cell comprising the steps of shooting a vehicle carrying said material into the cell.

Preferably the vehicle is the vehicle as hereinabove defined. Preferably the bullet is shot into the cell be means of a pressurised gas, for example helium.

The process of biological biolistics is often used where more standard techniques do not work. Resorbable impregnated materials, such as porous silicon offer biocompatible advantages over corrosion-resistant bulk metal materials.

According to a further aspect the present invention provides a method of making a vehicle for transferring material into a cell comprising the steps of rendering the vehicle at least partially porous and introducing to the vehicle the material to be transferred to the cell.

Preferably the vehicle comprises a silicon bullet, most preferably a submicron silicon particle, which may be rendered porous, preferably mesoporous by stain etching techniques. The bullet may have the material to be introduced to the cell adhered to it or alternatively it may be impregnated with the material.

The vehicle for transferring material into a cell may comprise bioactive silicon. It may comprise a bioactive silicon particle having the material to be transferred in a form adapted to co-precipitate with a substance which is taken up by cells. The co-precipitate may be a calcium phosphate precipitate.

According to another aspect the invention comprises a method of introducing material into a cell comprising associating the material with a silicon particle, precipitating calcium phosphate onto the particle to form a calcium phosphate/silicon particle combined particle, and arranging for the cell to uptake the calcium phosphate/silicon particle combination.

In the technique of electroporation the cell membrane can be made permeable by exposing cells to a brief electric shock of very high voltage. Low porosity bioactive silicon is electrically conducting and is suitably developed as an intimate coupling matrix for adherent mammalian cells growing on microelectrode arrays.

By having bioactive silicon, e.g. porous silicon or polycrystalline silicon, as one or both electrodes in electroporation apparatus it is envisaged that better DNA transfer takes place.

According to a further aspect the invention comprises a method of electroporation comprising providing an electrically conducting bioactive silicon electrode.

Preferably the method comprises growing cells on the electrode. The method may comprise providing an array of bioactive silicon electrodes, possibly with cells grown on them. The electrode, or electrodes, may be coated with porous silicon or may be of porous silicon throughout their cross-section, at least at a region of their height.

According to a further aspect the invention comprises electroporosis apparatus comprising a bioactive electrode. Preferably the electrode is bioactive silicon, most preferably porous silicon. An array of electrodes, or microelectrodes, may be provided.

The invention may also reside in the use of bioactive silicon, preferably porous silicon, in the preparation of apparatus for the introduction of materials into cells.

Several embodiments of the present invention will now be described by means of example only with reference to the Figures, in which:-

Figure 1 shows a partially porosified silicon microtip;

Figure 2 shows a silicon microneedle array;

Figure 3 shows the silicon microneedle array of Figure 2 having a macroporous network running from the tip to an underlying reservoir;

Figure 4 shows a porous silicon bullet impregnated with DNA;

Figure 5 shows a porous silicon core impregnated with DNA and surrounded by calcium phosphate; and

Figure 6 illustrates the electroporation technique of the present invention.

Figure 1 shows a micropiercer 10 in the form of a microtip 12 having a base width A of 50μm and a height B of 100μm and a tip width C of 0.5μm. The surface of the microtip 12 is coated with porous silicon 14 having a depth D of 0.1μm.

In use, the porous silicon coating 14 immobilises the substance to be delivered to the cell (e.g. DNA/RNA) on the tip itself, which increases the chances of the immobilised substance on the tip being introduced into the cell.

The pore size and porosity of the porous silicon coating can be controlled to tune the bioactivity of the microtip 12. By controlling the pore size and porosity of the porous silicon, we can make particular molecules come off it more, or less, readily. We may leave the microtips inside a cell for a predetermined time to allow molecules to disassociate themselves from the porous silicon.

Figure 2 shows an array 20 of silicon microneedles 22 extending away from a silicon support, or back, member 24. The microneedles 22 have porous silicon microtips 26 and a central lumen 28 communicating between the microtips 26 and a reservoir 30 defined between an upper member 32, provided with the microneedles 22 and the back, support, member 24. The back member 24 is of bulk silicon.

Figure 3 shows an array 33 of silicon microneedles 34 that is similar to that of Figure 2. The principal difference between the arrays shown in Figures 2 and 3 is that the microneedles 34 shown in Figure 3 are not provided with a central lumen 28. Instead the array 33 of silicon microneedles 34 in Figure 3 is provided with a mesoporous network 36 which extends from the microtips of the microneedles to the reservoir 30', allowing fluid communication between the reservoir 30' and the microtips.

In use, the substance to be delivered to cells is provided to the porous silicon microtips 22,34 from the reservoir 30,30' through the central lumens 28 or the mesoporous network 36. The substance is then held by the porous silicon microtips ready for introduction into a cell.

The material to be introduced into the cells may be pumped into the reservoir 30, 30', and out through the lumens 28 or porous network by a pump, not shown (but arrow 39 indicates the pump delivering liquid to the reservoir).

All or part of the silicon surfaces within the final structure may be treated in such a way as to modify their interaction with biological systems. This might be achieved by forming a layer of porous silicon on the surface. Such a layer could be formed by either an electrochemical

anodisation process or possibly by immersing the structure into a stain etching solution such as a mixture of hydrofluoric acid and nitric acid.

Figure 4 shows a biolistic bullet 40 comprising a submicron silicon particle rendered mesoporous by stain etching.

In use, the bullet 40 is impregnated with the substance to be introduced into a cell and is shot into the cell using pressurised helium. As the porous silicon is a resorbable material, it will be preferably fully resorbed, and at least partially resorbed, by the cell that it entered, and thus comprises less of a foreign body than known biolistic bullets such as gold or tungsten which leave particles of metal in the cell.

Figure 5 shows a porous silicon core 50 impregnated with a substance to be introduced into a cell (e.g. DNA/RNA) and calcium phosphate precipitate 52 formed around the core 50. The calcium phosphate 52 is co-pecipitated with DNA/RNA, so that a genetic material/calcium phosphate layer surrounds the bioactive silicon core 50. The bioactive silicon core locally induces calcium phosphate supersaturisation. It may be possible to place a bioactive silicon core next to a cell/against the wall of a cell, and co-precipitate DNA/$Ca(PO_4)_2$ against the core and against the wall of the cell. If the core is phagocytosed it can be resorbed.

The core 50 need not have DNA/RNA/any active substance on it - it may simply serve as a good nucleation site for co-precipitation of DNA/$Ca(PO_4)_2$.

It is known to use glass beads as a nucleation site for calcium phosphate co-precipitation DNA transfection - see for example the paper

by Watson and Latchman in "Methods (San Diego) 1996 10(3), 289-291 (Eng).

It will be appreciated that micropores are pores with a diameter of 2 nm or less; mesopores have a diameter of 2nm - 50 nm; and macropores have a diameter of 50 nm or more.

It has also been realised that it is possible to improve the efficiency of the introduction of materials to cells in an electroporation technique, as shown in Figure 6, using porous silicon, preferably mesoporous silicon (but macroporous and microporous silicon are also useful).

The use of a porous silicon (or porous other bioactive material, or bioactive polycrystalline silicon) electrode 60,61 achieves better performance in electroporation. Because the electrode is bioactive, instead of being bioinert, cells (typically animal cells) have an affinity to it and are localised on its surface.

Low porosity (50% or less, or 30% or less, or 10% or less) bioactive silicon is electrically conducting and is a suitable intimate complex matrix for adherent mammalian cells 62, which may grow on a microelectrode array 60,61. Thus, it is possible to grow mammalian cells on bioactive porous silicon electrodes and then introduce DNA (or other substances) into the cells by using electroporation, with the substrate upon which the cells are grown being an electrode, or even both electrodes 60,61, of the electroporation apparatus. This has advantages in handling the cells, and achieves a better efficiency rate of DNA introduction than solely having the cells suspended in a liquid medium 63.

The fact that porous silicon is resorbable/erodable in vivo in mammals has been proved by the inventors, and this underpins some aspects of the invention. The fact that silicon can be made bioactive underpins other aspects of the invention.

CLAIMS

1. A method of manufacturing a microneedle, or a microneedle array, the method comprising taking bulk silicon and creating a needle or an array of needles; and creating fluid transfer means extending from the base of the or each needle to its tip.

2. The method of claim 1 further comprising providing a network of pores from the base of the or each needle to its tip.

3. The method of claim 2 wherein the pores are macropores.

4. The method of claim 2 wherein the pores are mesopores.

5. The method of claim 2 wherein the pores are micropores.

6. The method of any preceding claim wherein the or each needle is created using photolithographic techniques.

7. The method of claim 6 wherein the photolithographic technique is anisotropic etching.

8. The method of claim 6 or claim 7 wherein the photolithographic technique is a photoresistant lithographic technique.

9. The method of any preceding claim wherein the bulk silicon wafer is an n-type substrate with a resistivity in the range of 0.1-10Ωcm.

10. The method of any preceding claim wherein the needle or needle array is planarised.

11. The method of claim 10 wherein a non-conducting mask is used to planarise the needle or array of needles.

12. The method of claims 10 and 11 wherein the planarised needle or array is treated so as to expose just the tips.

13. The method of claim 12 wherein the treatment uses an oxygen plasma treatment and an HF dip.

14. The method of any one of claims 10 to 13 wherein the tips are anodised to create pores from the tip to a wafer back surface.

15. The method of any preceding claim wherein a wafer provided with an array of tips is bonded to a backing member.

16. The method of claim 15 wherein the backing member and/or tip-carrying wafer is shaped so as to define a channel or reservoir between the tip-carrying wafer and the backing member.

17. A method of producing a micropiercer device comprising manufacturing one or more micropiercer projections, and providing substance holding means at or near the tip of the projections.

18. The method of claim 17 further comprising making at least a part of the projections porous.

19. The method of claims 17 or 18 further comprising making the tip of the projections porous.

20. The method of any one of claims 17 to 19 further comprising providing a porous coating on the tip.

21. The method of any one of claims 18 to 20 wherein the tip is made porous using an HF anodising technique.

22. The method of claim 18 comprising making substantially the entire extent of the tips porous.

23. A method of transferring a material into a cell comprising associating the material with a tip portion of a micropiercer and piercing the cell with the micropiercer.

24. The method of claim 23 further comprising using porous silicon to locate the material at or near the tip portion.

25. A method of genetic manipulation of a cell comprising associating genetic material with a tip portion of a micropiercer and piercing the cell with the micropiercer to allow the genetic material to enter the cell.

26. A microneedle array comprising a plurality of needles extending away from a support, the needles each having fluid transport means adapted to transport fluid from their bases to their tips, and fluid supply

means communicating with the fluid transport means and adapted to supply fluid to be injected to the base of the needles.

27. The array of claim 26 wherein the microneedles are made of silicon.

28. The array of claims 26 or 27 wherein the array is micromachined.

29. The array of claim 28 wherein the array is micromachined from a silicon wafer.

30. The array of any one of claims 26-29 wherein the fluid transport means comprises a reservoir.

31. The array of claim 30 wherein the reservoir extends under the needles.

32. The array of any one of claims 26 to 31 wherein the support has a lower portion, an upper portion, and a channel or reservoir extending between the upper and lower portions, with the needles being provided in the upper portion and the fluid transport means extending to the reservoir or channel.

33. The array of any one of claims 26 to 32 wherein the fluid transport means comprises a porous or capillary network provided in each needle.

34. The array of any one of claims 26 to 32 wherein each needle has a lumen extending through its longitudinal extent.

35. The method of claim 34 wherein the network comprises a plurality of mesopores.

36. The array of any one of claims 26 to 35 which is provided on an integrated silicon chip.

~~37. The method of claim 36 wherein the silicon chip has XXX.~~

37

38. A cell-penetrating member, or micropiercer, made of porous silicon.

38 37

39. The cell-penetrating member of claim 38 wherein the member is adapted to have a substance to be introduced into a cell carried by the porous silicon.

39

40. The cell-penetrating member of claim 42 wherein the porous silicon region immobilises a substance in comparison with its mobility when provided a bioinert substance such as titanium.

41 38 39

41. The cell-penetrating member of claims 39 or 40 wherein the porous silicon region is at the tip of the member.

41 38 40

42. The cell-penetrating member of claims 39 to 41 which has a tip or barb with no single central lumen.

42 38 40

43. The cell-penetrating member of claims 39 to 41 which has a needle with a central channel.

44. The cell-penetrating member of any one of claims 39 to 43 further having a pore network extending from a reservoir or channel to a substance delivery region provided on the surface of the micropiercer.

45. The cell-penetrating member of any one of claims 39 to 44 having a coating of porous silicon.

46. The cell-penetrating member of any one of claims 39 to 44 which is porous throughout its cross-section, at least at its tip.

47. The cell-penetrating member of any one of claims 39 to 46 which comprises a bulk silicon microtip with a porous silicon coating.

48. A vehicle for transferring material into a cell, the vehicle comprising at least, in part, resorbable material.

49. The vehicle of claim 48 wherein the vehicle comprises resorbable silicon.

50. The vehicle of claims 48 or 49 wherein the resorbable material is porous silicon.

51. The vehicle of claims 48 or 49 wherein the resorbable material is polycrystalline silicon.

52. The vehicle of claims 48 to 51 wherein the whole of the vehicle is made of the resorbable material, or only part of it.

53. The vehicle of claims 48 to 52 which comprises a biolistic bullet.

53

54. The vehicle of claim 52 wherein the bullet has a material to be introduced into a cell adhered to it.

54

55. The vehicle of claim 52 wherein the bullet is impregnated with material to be introduced into a cell.

55

56. The vehicle of claim 53 or 54 which is substantially saturated with material.

56

57. The vehicle of any one of claims 51 to 55 wherein the bullet comprises a submicron silicon particle.

57

58. The vehicle of claims 52 to 56 wherein the bullet has a porous coating.

58

59. The vehicle of any one of claims 52 to 57 which comprises bioactive silicon.

59

60. The vehicle of any one of claims 47 to 51 which has associated with it material to be transferred into a cell in a form adapted to co-precipitate with a substance which is taken up by cells.

60

61. The vehicle of claim 59 wherein the co-precipitate is a calcium phosphate co-precipitate.

61

62. A method of making a vehicle for transferring material into a cell comprising the steps of rendering the vehicle at least partially porous and introducing to it, or into it, the material to be transferred to the cell.

63. The method of claim 61 wherein the vehicle is a silicon bullet.

62 61

64. The method of claim 62 wherein the material to be transferred to the cell is adhered to the vehicle.

65. The method of claim 64 wherein the vehicle is a submicron particle and the material to be transferred to the cell is co-precipitated (with a precipitate substance), using the vehicle as a nucleation site.

66. The method of claim 62 wherein the vehicle is impregnated with the material to be transferred to the cell.

67. A method of electroporation comprising providing an electrically conducting bioactive silicon electrode.

68. A method according to claim 67 comprising growing cells on the electrode.

69. A method according to claim 67 or 68 comprising providing an array of bioactive silicon electrodes.

70. A method according to claim 69 comprising growing cells on the array.

71. Electroporation apparatus comprising a bioactive electrode.

72. Electroporation apparatus according to claim 71 in which the electrode comprises porous silicon.

73. Electroporation apparatus according to either of claims 71 or 72 having an array of electrodes, or microelectrodes.

73 74. A method of manufacturing the microneedle or microneedle array substantially as described herein with reference to Figure 2 or Figure 3 of the accompanying drawings.

74 75. A method of producing the micropiercing device substantially as described herein and with reference to Figure 1 of the accompanying drawings.

75
76. A cell penetration member substantially as described herein and with reference to Figure 1 or Figure 2 or Figure 3 of the accompanying drawings.

76
77. A vehicle for delivering material into a cell substantially as described herein with reference to Figure 4 or Figure 5 of the accompanying drawings.

77 78. A method of transferring material into a cell substantially as described herein with reference to Figure 4 or Figure 5 of the accompanying drawings.

78 79. A method of transferring material into a cell substantially as described herein with reference to Figure 6 of the accompanying drawings.

ABSTRACT

TRANSFERRING MATERIALS INTO CELLS AND A MICRONEEDLE ARRAY

The present invention relates to the use of porous silicon in the delivery of substances into cells. The porous silicon can be formed into micropiercers, microneedles and biolistic bullets for penetration of the cell. The control of the pore size and porosity of the porous silicon allows tuning of the bioactivity of the porous silicon. The porous silicon is also resorbable and is therefore resorbed from the cells without leaving any particles or being seen as a foreign body. The present invention also relates to the methods of manufacturing the porous silicon micropiercers, microneedles, microelectrodes, biolistic bullets, and precipitation of calcium phosphate on a bioactive substrate, and their advantages over known methods of delivering materials into cells.

To be accompanied, when published, by Figure 1 of the accompanying drawings.

Figure 1.

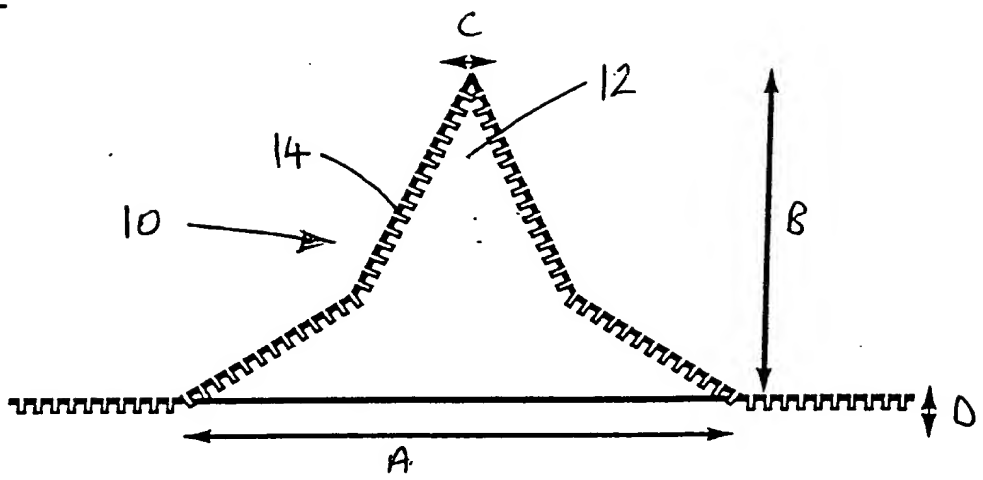


Figure 2.

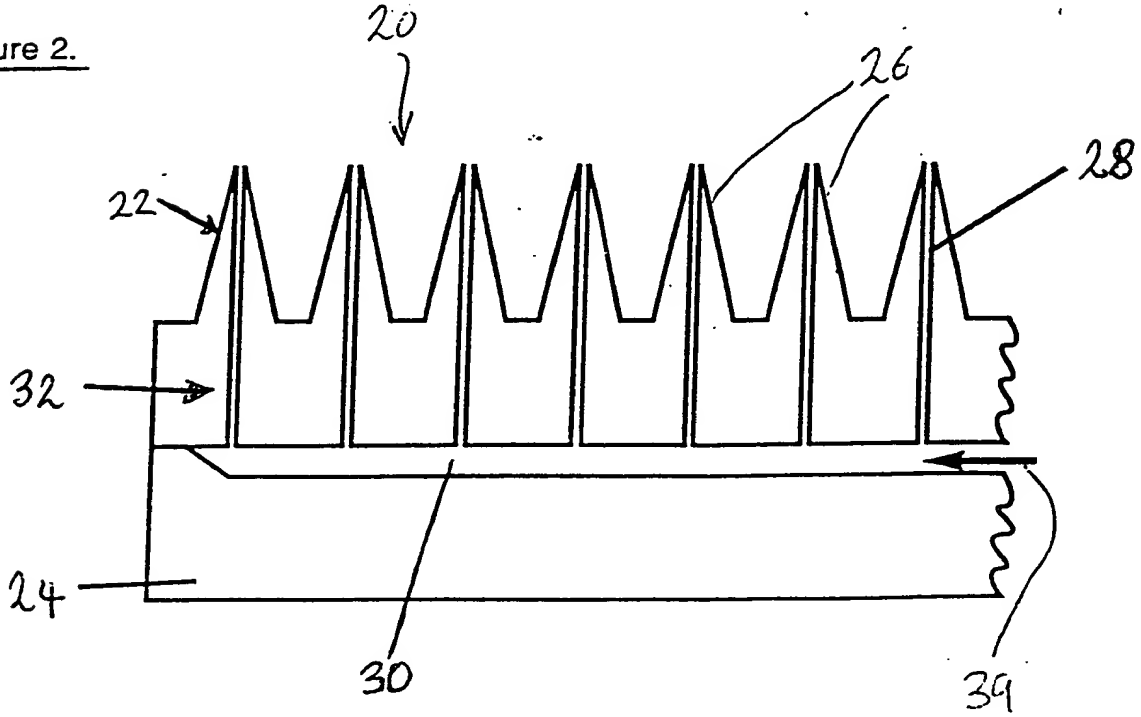


Figure 3.

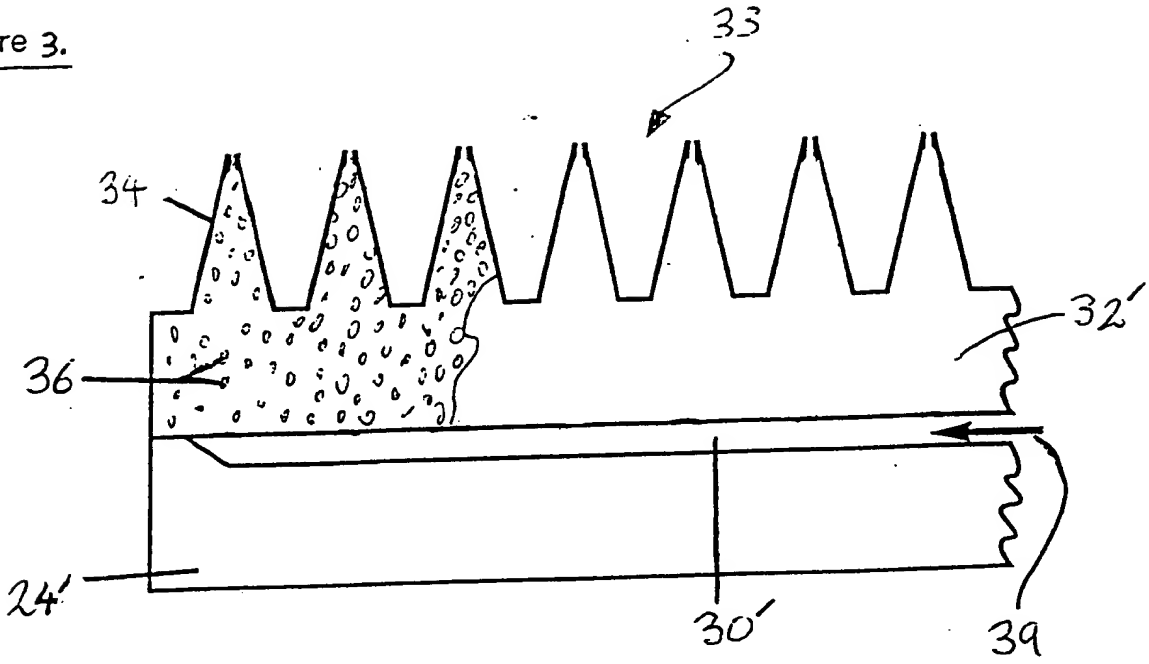
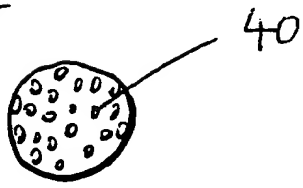


FIGURE 4.



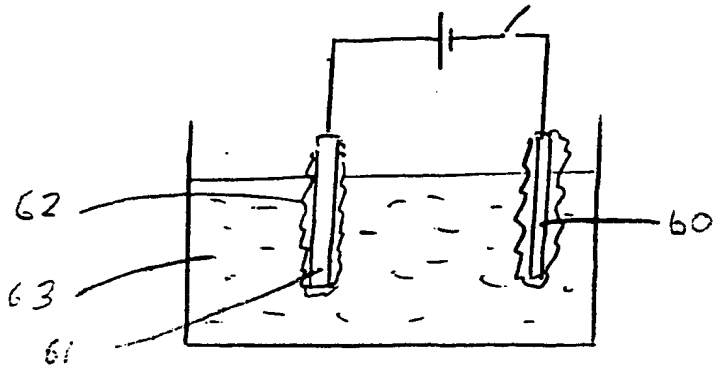


FIGURE 5.

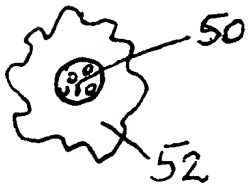


FIG. 6

DERA men

From: *Dr A W S Williams, IPD5B*

To: L. Canham *Date:*

Room: E4a11

This acknowledges receipt of your invention submission entitled:

DNA Transfer into Cells using Micromachining and Porous Silicon Technology

It has been date stamped with the date..... .....and will be dealt with as soon as possible.

Please keep me informed of further developments.

DR A W S WILLIAMS
T5
Ext: 4122

advantages with regard to the first 4 types of process listed as explained below.

DIRECT MICROINJECTION

This involves the insertion by a microneedle of DNA directly into the nucleus of individual cells. A glass micropipette linked to a micromanipulator is used to inject 10^{-8} - 10^{-7} µl into cell nuclei. 'Hits' are almost certain, given considerable operator expertise but the technique is laborious and cannot be applied to a large number of cells.

A recent US patent application (2) describes how an array of micromachined bulk Si tips could be employed to mechanically pierce large numbers of cells. With further development this method could be economical, precise and amenable to automation. Such microtip arrays have been under development in the past for field emission cathodes to be used in vacuum microelectronic applications. A 5 mm square chip will typically contain about 500 microtips of pyramidal shape with tip widths of 50 nm - 1 µm and heights of 10 - 100 µm, depending on process parameters chosen.

Invention Report on

DNA TRANSFER INTO CELLS USING MICROMACHINING AND POROUS SILICON TECHNOLOGY

One of the major underpinning processes in genetic engineering is that of introducing biological material, such as 'naked' DNA, into living cells. A variety of chemical and mechanical process have thus been developed, via which incorporation can be achieved (1). These include

- direct microinjection
- electroporation
- biolistics
- calcium phosphate method
- liposome, viral or bacterial mediated transformation
- protoplast fusion

A biocompatible Si technology-based approach would appear to offer major advantages with regard to the first 4 types of process listed as explained below.

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Two significant shortcomings of the invention (2) are (a) an ineffective means of transferring the biological material (eg DNA) into the pierced cell population (b) the stated need for the microtip array to be biocompatible material. It is for example proposed that the surface tension forces between closely spaced tips will enable the biological material to be trapped and stored between probes and substrate (2). It is also for example proposed that the microtips be used in-vivo during the course of surgical or endoscopic procedure as an adjunct in gene therapy.

A much more attractive approach is to combine such micromachining with anodisation techniques. This could yield

- bulk Si microtips with a porous coating that enables DNA immobilisation on the tip itself and tuneable bioactivity.
- Si microneedle arrays with either a single macropore or plurality of mesopores running from the tip to an underlying reservoir / capillary network in the chip.

Such structures are shown schematically in figures 1 and 2. Mesoporous field emission tips have already been realised by a few groups (3-5) and DNA fragments have been immobilised in macroporous silicon for flow-through genosensor applications (6).

The Si microneedle array concept of figure 2 is however, as far as I am aware, completely novel. It can be realised by, for example,

- standard anisotropic etching and lithographic techniques to generate tip array in 0.1 - 10 Ωcm n-type substrates.
- in-fill and planarisation using a non-conducting mask material.
- oxygen plasma treatment and HF dip to expose Si tips alone.
- anodisation to generate macropores from tip to wafer back surface.

ELECTROPORATION

The cell membrane can be made permeable by exposing cells to a brief electric shock of very high voltage. Low porosity bioactive silicon is electrically conducting and could be developed as the intimate coupling matrix for adherent mammalian cells growing on microelectrode arrays.

BIOLISTICS

The process of biological ballistics is used especially when the more standard techniques do not work for a particular cell line. It uses pressurised helium to shoot tungsten or gold microparticles coated with DNA into the cell.

Submicron Si particles are commercially available, relatively cheap and can be rendered mesoporous via stain etching techniques. Resorbable DNA impregnated material would at first sight seem to offer biocompatibility advantages over corrosion resistant bulk metallic materials.

CALCIUM PHOSPHATE METHOD

This is the method most commonly used to introduce DNA into cells. DNA uptake into cells can be aided by co-precipitation of DNA with calcium phosphate (7). The co-precipitate sediments onto cells and becomes adsorbed onto the membrane where it is taken up (phagocytosed) by those cells. A proportion of the DNA is then stably integrated into the nuclear genome. The mechanisms via which high transfection efficiency can be achieved are still under development, but for adherent mammalian cells it would seem a bioactive substrate that locally induces calcium phosphate supersaturation offers unique possibilities. To date I have only found literature on the use of bioinert substrate materials (8).

FUTURE WORK

A number of DNA transfer techniques have been identified that could benefit from 'bioSi' technology. Further paper studies are needed to select which technique modification to focus on. The fabrication of a prototype microneedle array however should be attempted in the near future, since this could have much broader applications with regard drug delivery in general, and perhaps even combinatorial chemistry.

(1) Principles of genetic manipulation: an introduction to genetic engineering. R. W. Old and S. B. Primrose, 5th edn. Blackwell (1994).

(2) Direct introduction of foreign material into cells. P.C.T. Applic. No. U595/12381.

(3) Field emission from pyramidal cathodes covered in porous silicon. P. R. Wilshaw et al. J. Vac. Sci. Techn. B12, 1 (1994).

(4) Fabrication of Si field emitters by forming porous silicon. D. Kim et al. J. Vac. Sci. Tech. B14, 1906 (1996).

(5) Porous silicon field emission cathode development. J. R. Jessing et al. J. Vac. Sci. Techn. B14, 1899 (1996).

(6) Advances in Genosensor Research. K. L. Beattie et al. Clin. Chem. 41, 700 (1995).

(7) Transformation of mammalian cells with genes from procaryotes and eucaryotes. Wigler et al. Cell 16, 777 (1979).

(8) Gene transfer of adherent mammalian cells using glass beads. K. Matthews et al. Mol. Biotechnol. 5, 259 (1996).

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